Characterization of Moroccan Population of *Erwinia Amylovora*, the Causal Agent of Fire Blight on Rosaceous

Afaf Ameur, My Mustapha Ennaji, Sophie Cesbron, Charles Manceau, Naima Rhallabi, and El Hassan Achbani

***Abstract*—Fire blight disease caused by the bacterium *Erwinia amylovora* is among the most serious threat to rosaceous, was introduced for the first time in Morocco in 2006. Since, the disease propagated then in wide causing immense damages. The objective of this work is the characterization of *E.amylovora* the population collected between 2006 and 2011. Characterization performed on a collection of 402 strains of *E.amylovora* from different regions of pome fruit. The obtained results demonstrated a big diversity of the Moroccan collection as well on the phenotypical and biochemical plan, the capacity of certain isolates to degrade the galactose, in particular of the hydrolyseesculin and appearance of colonies on MM2Cu and CCT media. Such as on the molecular plan in particular the ability of multiplex PCR analysis with PEANT1/2, pEA71F/R, Ea*Isc* F/R to identify the *E.amylovora* strains and the detection of absence of plasmid pEA29 in a few strains.**

***Index Terms*—*Erwinia amylovora*, rosaceous, biochemical and molecular characterization, Morocco.**

I. Introduction

Fire blight, caused by the Gram-negative bacterium *Erwinia amylovora* (Burrill, 1883) Winslow *et al*. is a very serious and destructive bacteria to pome fruit trees (pear, apple, quince, medlar) and many ornamental plants from rosaceous family (hawthorn, pyracanthe...). This disease manifested by the burn fruit, loss of limbs and affects the entire tree structure [1]-[3]. The disease described for the first time in 1780 in New York states, reported in 1794 [1], and introduced into Europe, first in England in 1957 and in Africa, first in Egypt in1964 [2]. In most countries, including the entire EU and EPPO region, *E.amylovora* regulate as a quarantine pathogen [4].

In 2006, the disease first observed in pear in Ain Orma, region of Meknes, Morocco [3]. The situation of this bacterium worsened every year through condition of climate; consequently other households have been identified infected.

Since the first apparition, fire blight has progressed most of rosaceous region affecting a total area of about 4000 ha causing serious economic losses menacing the national production of rosaceous plant.

The present study was to identify and characterize of a large collection of *E. amylovora* Moroccan strains, including those from the first foci in 2006 to the new breaks from 2011 and to determine the current situation of fire blight disease in this country.

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Achbani El Hassan is with the Plant Protection URPP- INRA-Meknes Morocco (e-mail: achbani105@gmail.com, dacus5@hotmail.com).

Afaf Ameur, My MustaphaEnnaji, and Naima Rhallabi are with the Laboratory of Virology, Hygiene, Microbiology and Quality/Eco-Toxicology and Biodiversity, Faculty of Sciences and Techniques, Hassan II Mohammedia University–Mohammedia, Casablanca, Morocco (e-mail: afaf.ameur@gmail.com, m.ennaji@yahoo.fr, rallabina@yahoo.fr).

Sophie Cesbron and Charles Manceau are with the Emersysequipe, Research Institute of Horticulture and Seed (IRHS), INRA Beaucouze, Angers, France (e-mail: sophie.cesbron@angers.inra.fr, charles.manceau@anses.fr).

II. Material and Methods

A. Strains and Samples

Samples from different parts of infected (different species) with various types of symptoms (shoot fire blight, necrosis of flower and leaf petioles and fruits) were collected from orchards in different rosaceous region. Diseased fragments were sterilized with hypochlorite of sodium (0.5%) for 2 minutes and washed twice in the Sterile Distilled Water (SDW). The fragments were dilacerated in a few drops of the SDW and allowed to stand for 20 minute more. 30 µL of the macerate obtained was inoculated (quadrant method) on LPGA medium. Petri dishes were incubated at 26 ± 1°C during 48h and the purification was performed on bacterial colonies which are similar to those of *E.amylovora* morphology (white, circular, mucoid, and curved) [5] Suspected colonies of *E. amylovora* were selected and further purified on LPGA at 26 ± 1°C. This operation was repeated three to four times to be sure that pure cultures were obtained for identification tests [6]. 402 Moroccan isolates and CFBP1430 as a reference strain obtained from collection CFBP, INRA Angers, France were added for comparison.

The strain of *E. amylovora* witness, CFBP 1430 was isolated in 1972 from a *Crataegus* sp. [7] and later used as a standard strain in many genetic and biochemical studies.

B. Biochemical Characterization

The characterization was based on the color, the characteristic aspect of the colonies of *E. amylovora* on semi-selective mediums such as CCT medium [8] and YDC, MM_2Cu [9]. and NBY (no growth at 36°C) [5] or differential like King'B medium [10].

Others tests were used [5]-[11]; as Hugh and Leifson [12], levan production and oxydase test [5]. The API 20^E system [13] was used for biochemical tests. The procedures of the manufacturer (BioMérieux) were followed in the preparation of the inoculums, the inoculation, the reading and the analysis of the results. Also, the isolated strains were tested for hypersensitive response (HR) on tobacco *Nicotian*

atabaccum var. *xanth i*leaves, according [11] using 10^8cfu/ml bacterial suspensions injected into the limb of a sheet of tobacco. After 24 hours, the checking the surface of the injection should show a lesion in the case of a plant pathogen organism. The type strain of *E. amylovora* CFBP 1430 was used as positive control and SDW as the negative one.

C. Pathogenicity Tests

The reproduction of the symptoms is carried out an apple seedling, having between 3 to 4 weeks, according to the technique described in [14]. The youngest leaf of plant is wounded by an incision with a scalpel infected of bacterial suspension (10^8cfu/ml). Two leaves were taken by apple seedling for this test and five replicates were used for each strain with CFBP1430 for a positive control and SDW for a negative control. The plants were kept in a humid chamber at 20–25℃ and observed twice during three weeks.

D. Molecular Characterization

The bacterial strains are identified by the Polymerize chain reaction (PCR) analysis was performed with a multiple xPCR: Peant1/2 (TATCCCTAAAAAACCTCAGT GC/GCAACCTTGTGCCCTTTA) [15], pEA71F/R (CCTGCATAAATCACCGCTGACAGCTCAATG/GCTAC CACTGATCGCTCGAATCAAATCGGC) [16] and EaIscF/R (CGCTAACAGCAGATCGCA/AAATACGCGCACGACC AT) [17]. The three primers specific selected of DNA of *Erwinia amylovora*, that two primers plamid's detecting pEA 29 and pEa71 and the third chromosome's DNA, first Each PCR mixture was carried out in a total volume of 20µl contained 5X PCR buffer GoTaq Flexi (4µl), 2.5 mM (1.5µl) of dNTP, 25mM MgCl2 (1.2 µl), 10µM each primer (0.25µl), 5U Taq (0.05µl) Polymerase (Promega, USA), and 50 ng of DNA template (1µl). The multiplex PCR was run at 95◦C for 1 minutes (1 cycle), 95◦C for 30 s, 60◦C for 30 s, 72◦C for 30 s (32 cycles), and 72◦C for 5 minutes (1 cycle).

The products were analyzed by electrophoresis in agarosegels at 1,8% with5X TAE buffer (w/v) and colored with Ethidium bromide for 20 minutes. An imaging system Bio Rad type is used for the revelation of the bands and taking pictures for the profiles obtained.

III. Results

A. Biochemical Characterization

During four years (2006-2011), 401 *E. amylovora* strains were isolated from six plants species affected and nine Moroccan regions, and compared them to the reference CFBP1430, France. The characteristics of strains are shown in Table I. We have characterized130*E. amylovora* strains of Moroccan population, isolated from affected hosts from *Pyrus* and *Malus* and seven Moroccan regions, and compared them to the CFBP1430 reference strains of this species from France. All these strains were negative Gram, non fluorescent on King's B medium, negative oxidase (except one strain, 1274-2), answering positively to tobacco hypersensibility test, were retained and their phenotypical and biochemical characteristics were compared.

TABLE I: Year of Isolation, Origin, Variety of Moroccan *Erwinia amylovora* Strains Studied

Year	Host	Nombre of strains	Origin			
2006	*Pyrus sp.*	2	Meknes			
2007	*Pyrus sp*	31	Agouray (18)	El Hajeb (13)		
2008	*Malus sp.*	2	Immouzar (2)			
				Taoujdtae		
	Pyrus sp.	23	Meknes (7)	(11)	El Hajeb (3)	Sefrou (2)
2009	*Malus sp.*	2	Azrou(1)	Sefrou(1)		
	Pyrus sp.	7	Meknes (5)	Azrou(2)		
	Cydonia sp.	2	Azrou			
2010	*Pyrus sp.*	234	Azrou (187)	El Hajeb (41)	Immouzzar (4)	Kenitra (2)
	Malus sp.	20	Azrou (15)	El Hajeb (4)	Immouzar (1)	
	Cydonia r sp.	34	Azrou (26)	El Hajeb (4)	Taoujdtae (4)	
2011	*Malus sp.*	11	Midelt(7)	Immouzar(2)	Azrou (2)	
						Meknes
	Pyrus sp.	15	Immouzar(8)	Midelt(4)	Azrou(2)	(1)
	Cydonia sp.	15	Immouzar(11)	Midelt(4)		
	Prunus sp.	2	Immouzar			
	Crateagus	1	Immouzar			

Colonies of these strains on CCT medium are a pale blue color with a particularly convex appearance and show an opaque edge with a clear and fine center, only one strain makes an exception, 1413-11, her aspect is different. On the MM2Cu medium containing the asparagin and copper sulfate, the color characteristic of the colonies of *E. amylovora* is yellow highly mucous after four days of incubation to 26 ± 1℃, except for three strains; 1272-10 (isolated in 2007) and 1413-11, 1417-7 and 1417-3 (2008) which express a cream-white color in this medium. On NBY, no growth is

observed at 36℃ except for 3 strains, 1413-11, 1414-4 and 1414-6, and on medium YDC separating *Pantoea* and *Erwinia* sp, the color expressed by the whole of the strains of *E. amylovora* is cream-white color. For the levan medium being used to check the polymerization of the fructose in polyfructose by the bacteria, the colonies are projecting and presenting an opaque marginal zone, characteristic of *E. amylovora*. Three strains showed their deficiency in levan, as 1266-14, 1274-2 (2007) and 1413-11 (2008) (Table II). All the strains are fermentative with slightly fermentative on the medium Hugh and Leifson.

TABLE II: *ERWINIA AMYLOVORA* STRAINS SHOWING ATYPICAL RESULTS IN COMPARISON WITH THE REFERENCE CFBP1430

Yea	Strains	Hypersensibilit	Levan	MM2Cu	CCT	NBY (36°c)	YDC
1972	CFBP1430	+	+	+	+	-	+
	the rest of strain						
2007	1272-10	+	+	-	+	-	+
	1266-14, 1272-2	+	-	+	+	-	+
2008	1413-11	+	-	-	-	+	+
	1414-6, 1416-4	+	+	+	+	+	+
	1417-7, 1417-3	+	+	-	+	-	+

Biochemical characterization of selected strains by API 20^E system revealed a great homogeneity, with 80% of the strains showing API 20^E profiles described in *E. amylovora*0005522 and 0007522.

In other words, all the strains tested form the acetone starting from the sodium pyruvate produce the acid after using glucose, the mannitol, the sorbitol, the sucrose and the arabinose. The liquefaction of the gelatin is variable; the 70% of the strains are unable to liquefy the gelatin.

B. Pathogenicity Tests

The pathogenicity was evaluated by symptoms of fire blight on inoculation leaves of apple seedling, 114 strains gave positive results with all *E. amylovora* strains assayed, except 16 strains which did not produce symptoms after two weeks on pear fruits as reported by other authors [18]-[19]. These leaves showed necrosis that started in the leaf wounds and progressed via the veins into the petioles, and droplets of ooze developed on the leaves. No symptoms were recorded in the case of leaves inoculated with SDW.

C. Molecular Characterization

Using PCR technique with plasmid and chromosomal primers: Peant1/2, pEA71F/R, Ea*Isc* F/R, amplified the bands of DNA of *E. amylovora* respectively 391pb, 105pb and 200bp, Fig. 1 shows the three bands of DNA. En effect, we have confirmed 401 *E. amylovora* strains isolated during 2006-2011.

All the strain reacted positively of a multiplex PCR excepted eleven Moroccan strains (1272-10, 1272-2, 1272-7, 1271-2, 1271-3, 1317-3, 1697-3,1720-5, 1733-6, 1732-5, 1878-1,) who didn't have a gene of plasmid pEA29. This plasmid participates of aggressively of *E.amylovora* strain [20], [21].

IV. DISCUSSION

After the first apparition of fire blight in Morocco in 2006 and the presence of *Erwinia amylovora* in most region production of pome fruit is presumably connected to several factors of development and could the absence of management and spread of bacteria. The implantation of efficient supervision and management of fire blight is essential. This study reports the identification of the Gram-negative *E. amylovora*, a causal agent of fire blight of rosaceous fruit and ornamental Maloïdeous cultivated either in full field or in the seedbeds, various tools were developed: 1) the use of the mediums (differential or semi-selective), 2) the biochemical tests, 3) the DNA hybridization and 4) the polymerase chain reaction (PCR). Several semi-selective mediums were proposed to detect *E. amylovora* such as the mediums CCT and MM2Cu used in our study and which are discriminating and conclusive, in agreement with former work [8].

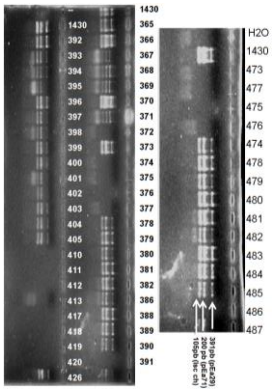

Fig. 1. PCR amplification of Moroccan *Erwinia amylovora* strains with the primers Peant, Pea71, and Ea Is.

The levan medium is generally used because of the characteristic convex colonies. Indeed, *E. amylovora* produces two types of exopolysaccharides (EPS), amylovoran and levan, which are the factors of virulence of *E. amylovora*. [22]. The levan is a polyfructan (b-2, 6-D-fructofuranan) synthesized by the levan sucrose secreted by several positive and negative Gram bacteria [23]. *E. amylovora* being in theory levan positive proved that its population can lodge defective individuals in this element as for 3 strains of our collection (1266-14, 1274-2 and 1413-11)

This result is in agreement with other reports in the bibliography which describe the presence of several levan-defective natural mutants as always having a functional

gene *lsc* [22]. This work shows that the Moroccan population of *E. amylovora* is highly diversified phenotypically and the use of certain mediums as "levan" will be able under considering the population of pathogen present in the sample and distorting even the result if the sample lodges only the germs levan negative. For the biochemical tests, the codes provided by Api 20^{E} system (0005522 and 0007522) generally are reported in the literature [13]-[18].

If these tests are heavy and require more than one week to identify the pathogen, the molecular tool, on the other hand, is fast and the result is already provided at 5 hours only for the conventional PCR and in less than one hour for the real time PCR. Mutiplex PCR analysis with the three primers (Peant1/2, *pEA*71F/R, Ea*Isc* F/R) was used for the first time as a molecular approach to identify of *E. amylovora* strains isolated in Morocco from different hosts and in different years. This analysis proved to be a sensitive, rapid and useful tool for discriminating among strains of this pathogen.

The *pEA*71F/R, EaIscF/R primers is present at all the population except the primer Peant1 /2 identify the plasmid *pEA*29 is unstable in our collection. On diagnosis, this result shows that only the primers as *pEA*71, EaIsc are reliable. In addition, other strain of fire blight reported absence of plasmid pEA29 [24], [25].

Otherwise, the pEA29 plasmid was detected in other E. *amylovora* strains [18]-[26]. The same case arises, other results reported when the primer Ea13di and Ea14rev is used in this population; third of its does not present [27]. The ability of Mutiplex PCR to detect *E.amylovora* strain, which is simpler and faster for the analysis of large numbers of strains, although it is less discriminating.

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REFERENCES

[1] J. L. Vanneste and S. Eden-Green, *Migration of Erwinia amylovora in host plant tissues In: Fire blight: The Disease and its Causative Agent, Erwinia amylovora*, Wallingford, UK: CABI Publishing, p. 370, 2000.

[2] T. V. derZwet, "Present worldwide distribution of fire blight. *ActaHorticulturae (ISHS)*," no. 590, pp. 33–34, 2002.

[3] E. H. Achbani, "Feu bactérien sur poirier au Maroc: Première apparition de cette brûlure bactérienne sur poirier dans la région de Meknès," *Phytoma La déf vég*, no. 606, pp. 26-28, July-August 2007.

[4] EPPO/CABI, *Quarantine Pests for Europe*, 2nd ed., CAB International, Wallingford (GB), 1996.

[5] N. W. Schaad, J. B. Jones, and W. Chun, *Laboratory Guide for identification of Plant Pathogenic Bacteria*, 3rd ed. St. Paul, MN: APS Press, 2001, p. 373.

[6] A. Jones and K. Geider, *II Gram negative bacteria. B. Erwinia* and *Pantoea. In: Guide for identification of plant pathogenic Bacteria*, 2nd edition, St Paul, USA: APS Press, 2001.

[7] J. P. Paulin and R. Samson, *Le feu bactérien en France, Il. Caractères des souches d'Erwinia arnylovora (Burril) Winslow et al*., vol. 5 no. 4, pp. 389-397, 1973.

[8] C. Ishimaru and E. J. Klos, "New medium for detecting *Erwinia amylovora* and its use in epidemiological studies," *Phytopathology*, vol. 74, no. 11, pp. 1342-1345, 1984.

[9] S. Bereswill, S. Jock, P. Bellemann, and K. Geider, **"**Identification of *Erwinia amylovora*by growth morphology on agar containing copper sulfate and by capsule staining with lectin," *Plant Dis*., no. 82, pp. 158-164,1998.

[10] E. O. King, M. Ward, and D. E. Raney, "Two simple media for the demonstration of pyocyanin and fluorescein," *J. Lab. Clin. Med*., no. 44, pp. 301-307, 1954.

[11] Z. Klement, "Rapid detection of the pathogenicity of phytopathogenic pseudomonads," Nature, no. 199, pp. 299-300, 1963.

[12] R. Hugh and E. Leifson "The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various gram negative bacteria," *J Bacteriol*, vol. 66, no. 1, pp. 24–26, July, 1953.

[13] R. Vantomme, C. Rijckaert, L.Verdonck, J. Mergaert, J. Swings, and J. D. Ley, "L'identificationd' *Erwinia amylovora*. *Revue de l'Agriculture*," no. 37, pp. 1158-1161, 1984.

[14] S. Cesbron, J. P. Paulin, M. Tharaud, M. A. Barny, and M. N. Brisset, "The alternative sigma factor HrpL negatively modulates the flagellar system in the phytopathogenic bacterium *Erwinia amylovora* under hrp-inducing conditions," *FEMS Microbiol Lett*., no. 257, pp. 221-227, 2006.

[15] P. Llop, A. Bonaterra, J. Peñalver, and M. M. Lopez, "Development of a highly sensitive nested-PCR procedure using a single closed tube for detection of *Erwinia amylovora* in asymptomatic plant material," *Appl. Environ. Microbiol*., no. 66, pp. 2071- 2078, 2000.

[16] R. K. Taylor, P. J. Guilford, R. G. Clark, C. N. Hale, R. L. S. Forster, "Detection of *Erwinia amylovora* in plant material using novel polymerase chain reaction (PCR) primers," *New Zealand Journal of Crop and Horticultural Science*, no. 29, pp. 35-44, 2001.

[17] V. Donat, E. G. Biosca, A. Rico, J. Peñalver, M. Borruel, D. Berra, T. Basterretxea, J. Murillo, and M. M. López, "*Erwinia amylovora* strains from outbreaks of fire blight in Spain: phenotypic characteristics," *Annals of Applied Biology*, no. 146, pp. 105–114, 2005.

[18] S. M. Lehman, W. S. Kim, A. J. Castle, and M. Svircev, "Duplex real-time polymerase chain reaction reveals competition between *Erwinia amylovora* and *E. pyrifoliae* on pear blossoms," *Phytopathology*, no. 98, pp. 673–679, 2008.

[19] E. Billing, L. A. Baker, J. E. rosse, and C. M. E. Garrett, "Characteristic of English isolates of *Erwinia amylovora* (Burrill) Winslow *et al*.," *Journal of Applied Bacteriology*, no. 24, pp. 195–211, 1961.

[20] H. Falkenstein, W. Zeller, and K. Geider, "The 29 kb plasmid, common in strains of *Erwinia amylovora*, modulates development of fire blight symptoms," *J Gen Microbiol*., no. 135, pp. 2643–2650, 1989.

[21] J. Laurent, M. A. Barny, A. Kotoujansky, P. Dufriche, and J. Vanneste, "Characterization of a ubiquitous plasmid in *Erwinia amylovora*," *Mol Plant Microbe Interact***,** no. 2, pp. 160–164, 1989.

[22] G. Geier and K. Geider, "Characterization and influence on virulence of the levansucrase gene from the fire blight pathogen *Erwinia amylovora*," *Physiological and Molecular Plant Pathology*, no. 42, pp. 387-404, 1993.

[23] P. Bellemann and K. Geider, "Localization of transposon insertions in pathogenicity mutants of *Erwinia amylovora* and their biochemical characterization," *Journal of General Microbiology*, no. 138, pp. 931-940, 1992.

[24] P. Llop, V. Donat, M. Rodríguez, J. Cabrefiga, L. Ruz, J. L. Palomo, E. Montesinos, and M. M. López, "An indigenous virulent strain of *Erwinia amylovora* lacking the ubiquitous plasmid pEA29," *Phytopathology*, no. 96, pp. 900–907, 2006.

[25] R. A. Mann, T. H. M. Smits *et al*., "Comparative genomics of 12 strains of *Erwinia amylovora* identifies a pan-genome with a large conserved core," *PLoS ONE*, vol. 8, p. e55644, 2013.

[26] P. Lecomte, C. Manceau, J. P. Paulin, and M. Keck, "Identification by PCR analysis on plasmid pEA29 of isolates of *Erwinia amylovora* responsible of an outbreak in Central Europe.Eur," *J. Plant Pathol*., no. 103, pp. 91–98, 1997.

[27] E. H Achbani., A. Benbouaza, N. Hannou, and M. Moumni, "Caractérisation de la population marocaine d'*Erwinia amylovora*, agent causal du feu bactérien des rosacées fruitières à pépins," in *Proc. 7th AMPP*, 2010, pp. 30-38.

Afaf Ameur was born in Morocco, on January 3rd 1989. She graduated with Agricultural Master's degree in Plant and Environment Protection from National School of Agriculture of Meknes (ENAM) in 2011. Now, she is preparing a Phd in Faculty of Sciences and technology Mohammedia, Hassan II-Mohammedia-University-Morocco in cooperation with Plant Protection URPP- INRA-Meknes Morocco. The main research is focusing on Molecular biology and biocontrol of plant diseases.